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(71) Applicant *(for all designated States except US)*: MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB).

(71)(72) Applicants and Inventors: CREIGHTON, Andrew, Malcolm [GB/GB]; 68 Millway, Mill Hill, London NW7 3QY (GB). BRITTON, Keith, Eric [GB/GB]; 122 Hertford Road, De Beauvoir Town, London N1 4LP (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: NERI, Dario [IT/GB]; 15 Hayster Drive, Cherryhinton, Cambridge CB1 4PB (GB). PETRUL, Heike [DE/DE]; Ulrich-Pfeiffer Strasse 8, D-88518 Herbertingen (DE).

(74) Agent: LEAROYD, Stephanie, Anne; British Technology Group Limited, Patents Dept., 101 Newington Causeway, London SE1 6BU (GB).

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(54) Title: RADIOLABELLED PROTEINS

(57) Abstract

A structurally modified binding protein wherein the binding protein is fused to a peptide sequence capable of acting as a substrate for a casein kinase II enzyme.

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RADIOLABELLED PROTEINS

BACKGROUND TO THE INVENTION

Field of the invention

This invention is in the field of radiolabelled proteins, methods for their preparation and their use in radioimmunoassays and radioimmunotherapy. The term "protein" as used herein includes polypeptides.

Description of the prior art

Although radioimmunoscintigraphy is becoming routinely available in nuclear medicine and plays an increasingly important role in tumour diagnosis, radioimmunotherapy i.e., labelling antibodies or other targeting molecules particularly with ^{32}P, is still in its infancy. For successful radioimmunotherapy it is essential that an adequate dose of the labelled antibody reaches, binds to and remains attached to the tumour tissue and that only minimal amounts become bound to normal, non-tumour tissue. The effective delivered dose of radioactivity depends not only on the amount taken up and its residence time but also on the physical half-life, the abundance of the emission, its energy and its physical relationship with the biocidal activity.

Howell *et al.*, 1994, J. Nucl. Med., 35, 1861-1869 report that of several radionuclides, ^{32}P is one of the most promising. The use of a high energy, relatively long range β- emitter such as ^{32}P would allow the destruction of tumour cells not expressing the target antigen that are within a few millimetres of those taking up the ^{32}P- labelled antibody. ^{32}P also has the appropriate energy and the fairly long half-life ensures that a tumouricidal dose is achieved. Since whole antibodies which are bound to tumour have long residence times, they allow fairly long half-lived nuclides to be used to ensure adequate doses. In summary, long residence time on the tumour and rapid clearance from the normal tissues will give successful radioimmunotherapy.

Several procedures have been described for labelling monoclonal antibodies and other proteins with ^{32}P. For example, UK Patent GB-B-2,186,579 describes a system for modifying a protein that will bind with a tumour-associated structure comprising the introduction into the binding protein of a peptide region which is capable of acting as a substrate for a phosphokinase. The resulting modified binding protein can then be

^{32}P-labelled by reacting it with a ^{32}P-labelled gamma nucleoside triphosphate in the presence of a phosphokinase.

Other procedures have been described in UK Patent Application GB-A-2,262,528 wherein using conventional peptide chemistry, a phosphorylatable conjugate is produced in which the substrate molecule is directly bonded through its carboxy terminus through an amide link to the targeting molecule (for example an antibody).

All the prior art thus relates to the chemical coupling of the phosphorylable substrate to the antibody or other binding protein. Useful, high-affinity recombinant antibodies can now be isolated from libraries. It has however, been a problem to produce products for use in ^{32}P-radioimmunotherapy by techniques of genetic engineering, using recombinant antibodies or other targeting proteins, since fusion of cyclic AMP-dependent kinase peptidic substrates such as described above is unsatisfactory because of excessive tendency to proteolysis in over-expression systems for soluble antibodies. The products can readily lose their tag during bacterial expression due to enhanced susceptibility to proteolysis that can be attributed to the presence of positively charged arginine residues in the phosphokinase substrate molecule.

Further prior art is discussed after the "Summary of the invention", without which its context would not be clear.

Summary of the invention

It has now been found that efficient and site-specific antibody phosphorylation of recombinant antibodies with ^{32}P can be achieved using casein kinase II peptidic substrate sequences linked (meaning attached or tagged - generally described as fused at the gene level)) to the antibody. These substrates are particularly stable. It has been found that the antibodies phosphorylated following this strategy are stable in human plasma for 48 hours as 37^{o}C and retain full immunoreactivity. Additionally, this labelling strategy confers several negative charges to the recombinant antibodies, improving their behaviour in polyacrylamide gel electrophoresis and allowing the study of antibody-antigen interaction by gel retardation analysis. It is envisaged that the invention will be of use with any binding protein, not just antibodies.

Accordingly, the invention provides a structurally modified binding protein wherein the binding protein is fused to a peptide sequence capable of acting as a substrate for a

casein kinase II enzyme.

Further Description of the Prior Art

European Patent Application Number 0 372 707 (Pestka) describes modified proteins, principally interferons, which can be phosphorylated. In this patent application, Pestka describes the modification of proteins, specifically human interferons, which have proven to be non-phosphorylatable by cyclic AMP(cAMP)-dependent protein kinases. He proposes the modification of such proteins by the addition to that protein of a phosphorylatable site and dsecribes these as "modified" proteins. DNA sequences encoding such modified proteins are described. The modified proteins may then be phosphorylated using a protein kinase. Pestka then lists a number of types of protein kinase enzymes including for example cAMP-dependent, cyclic GMP dependent, cyclic nucleotide-independent kinases and casein kinases I and II. All are purported to be useful in the invention.

However, the present inventors have noted that it is not possible to modify a recombinant antibody to introduce any phosphorylatable site for reaction with any protein kinase. For secreted recombinant antibodies, as discussed above, the fusion of cyclic-AMP dependent kinase substrates to antibodies is unsuccessful due to excessive tendencies to proteolysis of such antibodies during secretion, and hence phosphorylation is not possible.

Hence Pestka is non-enabling for secreted proteins; a skilled person would need to conduct extensive research to sort out suitable methods for individual cases following the teaching in EP 0372707. As such, Pestka is merely an invitation to the skilled person to embark on a research project.

Description of the preferred embodiments

Casein kinase II is a protein serine/threonine kinase having activity that is independent of cyclic nucleotides and calcium and has been shown to phosphorylate many different proteins. It is often called a multifunctional protein kinase.

Casein kinase II peptide substrates have been described by Marin *et al.* 1986, Eur. J. Biochem., 160, 239-244 and Kuenzel *et al.* 1987, J. Biol. Chem. 262, 9136-9140. However, they have never been proposed as tags for protein phosphorylation and thus the structurally modified binding proteins (referred to hereinafter as constructs) of the present

invention are believed to be novel.

Casein kinase II substrates contain phosphorylation sites. A common feature of these sites is that they are located in sequences containing several amino acids with acidic side chain groups.

In the present invention, preferred casein kinase II substrates include both synthetic peptides and incorporated protein structures such as calmodulin which possess the appropriate sequences.

Preferred casein kinase II substrate sequences for use in the present invention contain at least one phosphorylatable residue and at least two negatively charged residues to either the left (NH_2 terminal) or right (COOH terminal) or left and right of the phosphorylatable residue less than 5 or 5 residues spacing away from the phosphorylatable residue and are 5 to 20 residues in length. The phosphorylatable residue is, preferably threonine or more preferably serine. Of course any combination of serine and threonine may be used where there is more than one phosphorylatable residue present, but preferably, serine is used in all cases. It has been found that the efficiency of phosphorylation of the phosphorylatable residue is increased by the presence of the negative charges, aspartate and glutamate. Thus any casein kinase II peptide substrate which contains a phosphorylatable residue and at least two negative charges, preferably glutamate or more preferably aspartate residues in the specified positions relative to the phosphorylatable residue may be used.

Furthermore, several naturally occurring casein kinase II substrates contain more than one phosphorylatable serine residue in a row, which, upon phosphorylation contribute to the negative charge of the kinase substrate and to the efficiency of phosphorylation.

Thus other preferred peptides of the present invention for use as casein kinase II substrates contain more than one phosphorylatable serine residue together with negatively charged residues including phosphorylated serine, preferably glutamate or more preferably aspartate residues.

The negative charged residues including phosphorylated serine are preferably 5 or less than 5 residues away from the phosphorylatable residue. Preferably they are 3 and 1 or 5 residues away from the phosphorylatable residue and more preferably on the COOH terminal alone or more preferably on both the COOH and NH_2 terminals. More preferably, a cluster of negatively charged residues is used after and/or before the residue

to be phosphorylated. The peptide is preferably 5-15 residues long. Particularly preferred are peptides in which there is a negatively charged residue to the left (NH_2) of the phosphorylatable residue(s) which is 3 residues spacing away and a negatively charged residue to the right (COOH) of the phosphorylatable residue(s) which is 5 residues spacing away.

Peptide sequences useful in the present invention are, for example, described in Kuenzel *et al.*, J. Biol. Chem. 1987, 262 9136-9140.

Particularly preferred peptides have the following sequences

DDSDDDEE (SEQUENCE ID NO 1)

DDDSDDD (SEQUENCE ID NO 2)

These highly negatively charged casein kinase II substrates are advantageous since as well as being efficiently phosphorylated they are easily and efficiently purified by ion exchange chromatography and have good stability and expression levels when tagged to recombinant antibodies.

The binding proteins of the invention are preferably antibodies or antigen-binding antibody fragments including preferably monoclonal antibodies or antigen-binding fragments thereof. Preferably, where the labelled antibody is destined for a therapeutic application, this antibody is a humanised monoclonal antibody or an antibody fragment. Where the labelled antibody is destined for a non-therapeutic application, it may be a polyclonal but more preferably a monoclonal antibody. The choice of binding protein will depend on the eventual use of the binding protein and the antigen to which it is directed and will therefore be apparent to a skilled person.

The binding protein will normally be a monoclonal antibody that will bind with a tumour associated antigen, for example antigens associated with solid tumours with relatively poor blood supplies.

More generally, the binding protein may be any protein that will bind with a tumour-associated protein or other tumour-associated structure such as a glycolipid or carbohydrate, where the tumour is one susceptible to high energy beta particles and, in addition to monoclonal antibodies, the targeting protein could be, for example, a fragment of an antibody for example scFv or a hormone or similar peptide that will bind to an appropriate receptor site identified on certain types of tumour cell, e.g. melanocyte-

stimulating hormone, epithelial growth factor, interferons and mitogenic peptides such as bombesin.

Antibodies to be tagged with the peptide will depend on the tumour to be targeted or on the eventual use of the construct in immunoassay and thus the choice of antibody will be apparent to a person skilled in the art. It is also envisaged that in addition to antibodies, other binding molecules such as proteins, peptides, drugs, biotin and oligosaccharrides can be tagged by a casein kinase substrate peptide of the present invention.

If the construct is to be used in therapy, then the recombinant antibody will preferably be a monoclonal antibody which will bind to a tumour-associated antigen. For example, antigens associated with solid tumours with relatively low blood supplies. Such said tumours include those found in the colon, ovaries and lungs and monoclonal antibodies to such tumour-associated antigens are already known and have already been used as delivery vehicles for other anti-tumour agents. Such known antibodies can be linked to ^{32}P by the techniques of the current invention. More preferably the recombinant monoclonal antibody is humanised, or is an antigen-binding antibody fragment (Winter & Milstein, 1991, Nature, 349, 293-299). The smaller size of antibody fragments such as Fab, scFv and Fv fragments results in faster blood clearance and lower immunogenicity.

For the purposes of an experimental demonstration of the benefits of the present invention, work is done with monoclonal antibodies usable in rat and mouse experimental systems.

The casein kinase II substrate sequences may be attached at the linked region (between VH and VL) and/or at one C-terminal end of the antibody. The amount of casein kinase II substrate or substrates added and thus the eventual amount of ^{32}P label depends on the final use of the antibody and is thus apparent to a person skilled in the art, whether he is a medical practitioner treating a patient with a tumour or a laboratory assistant performing a radioimmunoassay.

Linkage or tagging of the casein kinase II substrate to the antibody or other protein is achieved at the gene level by the fusion of the antibody or other protein and one of the substrate genes. This technique is described in Example 1 hereinafter.

Labelling of the thus formed structurally modified binding protein is simple. For example it can be achieved by mixing the phosphorylatable binding protein, casein

kinase 2 and gamma ^{32}P-ATP in a phosphorylation buffer at 20°C for 15-30 minutes.

Once the substrate peptide has been introduced into the targeting protein, it can be phosphorylated or thiophosphorylated to introduce ^{32}P. The phosphorylation can be carried out by procedures known per se and by procedures which are described for example in British Patent GB-B,2,186,579, although with the cyclic AMP dependent kinase. The phosphorylation is normally carried out by using gamma-^{32}P-adenosine triphosphate (gamma-^{32}P-ATP); or using gamma-^{32}P guanosine triphosphate, in the presence of a casein kinase, which brings about the labelling with ^{32}P of the serine or threonine residue in the substrate peptide. Although the serine-containing peptides can normally be phosphorylated very rapidly at 37°C, or more conveniently at room temperature, the threonine containing peptides usually require a longer time and it is necessary to reduce the temperature of the incubation to maintain the stability of the enzyme and the substrate construct of the invention. Conveniently these labellings are carried out at 10°C overnight although these conditions are not optimal.

The phosphorylation of the substrate peptide portion of the structurally modified protein of the invention is normally carried out shortly prior to the clinical use of the labelled conjugate but the labelled conjugates are reasonably stable and can normally be stored prior to their clinical use.

As an alternative to phosphorylation, the structurally-modified targeting proteins of the invention can be thiophosphorylated by methods known per se, e.g. those disclosed in WO90/11289.

Once the phosphorylation of the structurally-modified targeting proteins has been completed, the ^{32}P labelled conjugate can be purified by standard chromatographic techniques such as gel filtration, e.g. on a Sephadex^{R} column equilibrated with phosphate buffered saline. The ^{32}P conjugate solution obtained in this way may then be filtered, e.g. using a 0.22 μm pore size filter so that it is in a suitable form for clinical use.

The tagged constructs of the invention can alternatively be purified from unreacted ^{32}P by affinity chromatography. For example, some of the constructs allow the production of antibodies that can be affinity purified with anti-FLAG columns (DN227, DN250, DN255 - see Example 1 below; column commercially available from Kodak Inc.) or with anti-myc tag columns (DN232, DN249- see Example 1 below): (Marks *et al.*, 1991,

J. Mol. Biol., 222, 581-597). These methodologies exploit the fact that the relevant antibodies contain, in addition to one or more phosphorylation sites, a peptidic tag which can be recognised by specific monoclonal antibodies.

Additionally, calmodulin can be fused to the antibodies described herein above. Recombinant antibodies fused to calmodulin can efficiently be purified on calmodulin antagonist column (D. Neri, C. de Lalla, H. Petrul, P. Neri, G. Winter, 1994 "Calmodulin as a versatile tag for antibody fragments". Submitted to *Bio/Technology*). Calmodulin is a small protein, non-immunogenic and therefore suitable for these applications.

The constructs of the invention provide particular use in radioimmunotherapy and thus the invention further provides a construct according to the invention for use in therapy.

When used in therapy, the construct may be formulated in a composition. Accordingly the invention further provides a pharmaceutical composition particularly one for parenteral administration comprising a construct of the invention in association with a pharmaceutically acceptable diluent or carrier.

Once a trace dose of the radiolabelled binding protein is shown to target preferentially for a tumour as compared to normal tissue, then the ^{32}P-labelled construct may be given to the subject intravenously or into various body regions, e.g. by intraperitoneal, intrapleural or intra-arterial infusion.

The invention further provides the use of a construct described above in the manufacture of a medicament for the treatment of a patient in need of radioimmunotherapy.

The invention further provides a method of treatment of a patient in need of radioimmunotherapy comprising the administration of a therapeutically effective amount of a construct of the present invention.

The constructs of the present invention also find uses in analytical applications such as antibody biodistribution experiments, development of radioimmunoassays and the determination of antibody-antigen affinity constants.

^{32}P labelled recombinant antibodies retain full immunoreactivity, since the labelling occurs at defined sites that do not interfere with antigen binding. Since extremely high specific activities can be achieved, one expects that ^{32}P-labelled recombinant antibodies will be invaluable for new one- or multi-step radioimmunoassays. The labelled recombinant antibodies of the present invention, directed to the targets of the

immunoassays, can be used directly, without the need of a labelled secondary antibody. Experimental times of radioimmunoassays are therefore expected to become shortened. Furthermore, the use of ^{32}P instead of iodine is advantageous for safety reasons.

Additionally, for both therapeutic and non-therapeutic applications, the recombinant antibodies may be labelled with ^{33}P. ^{33}P produces weaker β-emission than ^{32}P thus in non-therapeutic applications enabling the protective screening in the laboratory to be dispensed with. Its longer half-life than ^{32}P can also be convenient in a laboratory setting. In therapy, the longer half life of the β emission and shorter radius of action of ^{33}P may in some situations be advantageous.

High-sensitivity detection of recombinant proteins is becoming more and more important in chemical and biological laboratory practice. For example, labelled proteins can be used to screen libraries, or to detect minute amounts of proteins in gels. In the case of recombinant antibodies, we have shown that high-sensitivity detection of proteins in gels allows the determination of antibody-antigen affinity constants by gel retardation assays (Neri *et al.*, 1994, J. Mol. Biol., 246 , 367-373).

The invention will now be illustrated by way of the following Examples with reference to the drawings in which:

Figure 1 shows a schematic representation of the expression vectors for the production of phosphorylatable antibodies. rbs. ribosome binding site; PelB, leader peptide; VH and VL, variable genes of the antilysozyme antibody D1.3; myc, EQKLISEEDLNGAA (SEQUENCE ID NO 3) (Munro and Pelham, 1986); flag, DYKDDDDK (SEQUENCE ID NO 4) (Hopp *et al.*, 1988); stop, two stop codons. The position of relevant restriction sites is indicated. pDN249 is the same as pDN232, but the VH and VL genes are those of the antilysozyme HyHEL-10 antibody. Putative phosphorylation sites are indicated as underlined serine residues (S). pDN255 is scFv(HyHEL-10) with (Gly4Ser) linker, cloned into the Sfi1/Not1 sites of pDN227.

Figure 2 shows a purification profile of ^{32}P-DN255.

EXAMPLE 1: Cloning of vectors for antibody phosphorylation

Some of the constructs in this Example are shown schematically in Figure 1. Vector construction was performed according to standard molecular biology techniques (Sambrook *et al.*, 1990, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

pDN30

A DNA fragment coding for the cAMP-dependent protein kinase substrate RRASL, the myc tag (Munro and Pelham, 1986, Cell, 46, 291-300) and two stop codons was PCR amplified from pUC1198SNpolymyc (Figini *et al.*, 1994, J. Mol. Biol., 239, 68-78) using the primers NOTKINMYC (SEQUENCE ID NO 5) (5' GTC CTC GCA ACT GCG GCC GCA AGA AGG GCA AGT GTT GAA CAA AAA CTC ATC TCA GAA 3') and LMB2 (SEQUENCE ID NO 6) (5' GTA AAA ACGA CGG CCA GT 3'). The fragment was gel-purified, EcoR1/Not1 digested and cloned into EcoR1/Not1 digested pDN5, an expression plasmid containing the anti-lysozyme scFv(D1.3) cloned in pUC119SNpolymyc (plasmid "pDN5"; Neri *et al.*, 1995, J. Mol. Biol., 246, 367-373). The correctness of the cloning was checked by ELISA, production and purification of the antibody on a lysozyme-sepharose column (Neri *et al.*, 1995, J. Mol. Biol., 246, 367-373) and DNA sequencing of the region of the plasmid containing the Not1 and EcoR1 sites.

pDN31

A DNA fragment coding for the cAMP-dependent protein kinase substrate RRASL, a seven aminoacid spacer terminating with a cysteine residue, the myc tag (Munro and Pelham. 1986) and two stop codons was PCR amplified from pDN23 (Neri *et al.*, J. Mol. Biol., 246, 367-373) using the primers NOTKINCYS (SEQUENCE ID NO 7) (5' GTC CTC GCA ACT GCG GCC GCA AGA AGG GCA AGT GTT GGC GGT TCT TCC GGC TCC 3') and LMB2 (SEQUENCE ID NO 6) (5' GTA AAAA CGA CGG CCA GT 3'). pDN23 is a derivative of pHEN1 (Hoogenboom *et al.*, 1991, Nucleic Acids Res., 19, 4133-4137). which does not contain the phage gene III and in which the sequence between the Not1 and the EcoR1 sites (underlined) has been replaced by the sequence shown below:

GCGGCCGCAGGCGGTTCTTCCGGCTCCTGTGAACAAAAACTCATCTCAGAAG AGGATCTGAATTAATAAGAATTC, (SEQUENCE ID NO 8)

The PCR-amplified fragment was gel-purified, EcoR1/Not1 digested and cloned into EcoR1/Not1 digested pDN5. The correctness of the cloning was checked as for pDN30.

pDN223

The gene of the anti-lysozyme scFv(D1.3) was amplified and cloned in pUC119SN polymyc (plasmid "pDN5"; Neri *et al.*, 1995, J. Mol. Biol., 246, 367-373) by PCR using the primers LMB3 (SEQUENCE ID NO 9) (5' CAG GAA ACA GCT ATG AC 3') and CASEPORI (SEQUENCE ID NO 10) (5' TGA CTG GAA TTC TTA TTA TTC TTC ATC GTC GTC GGA ATC GTC ATC TGC GGC CGC CCG TTT GAT CTC GAG 3'). The resulting fragment was gel-purified, digested with EcoR1/Sfil and cloned into EcoR1/Sfil digested pDN22 (Neri *et al.*, 1995, J. Mol. Biol., 246, 367-373).

pDN227

The phosphorylatable antibody gene was obtained by PCR using the primers LMB3 and CASEFOR2 (SEQUENCE ID NO 11) (5' TGA CTG GAA TTC TTA TTA CTT GTC ATC GTC GTC CTT GTA GTC ATC GTC GTC GGA ATC GTC ATC TGC 3') and pDN223 as template. The product was gel-purified and cloned into EcoR1/Sfil digested pDN22 (Neri *et al.*, 1995, J. Mol. Biol., 246, 367-373).

pDN232

The complementary oligonucleotides PHOSPHOLINK1 (SEQUENCE ID NO 12) (5' GTC ACC GTC TCC TCA GAC GAT GAC TCT TCC TCT GAT GAC GAT TCT GAC GAA GAC ATC GAG CT 3') and PHOSPHOLINK1FOR (SEQUENCE ID NO 13) (5' CGA TGT CTT CGT CAG AAT CGT CAT CAG AGG AAG AGT CAT CGT CTG AGG AGA CG 3') were kinased and annealed using standard techniques (Sambrook *et al.*, 1990. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY, then ligated into BstE2/Sac1 digested pDN5.

pDN249

The VH and VL genes of the anti-lysozyme antibody HyHEL-10 (Lavoie *et al.*, 1992, J. Immunol., 148, 503-513) were PCR amplified as described (Clackson *et al.*, 1991, Nature (London), 352, 624-628) and digested and cloned sequentially into the SfiI/BstE2 and Sac1/EcoR1 sites of pDN232, respectively.

pDN250

In this construct, casein kinase II sequences are incorporated both at the linker and the C-terminal position.

pDN232 was SfiI/Not1 digested; the resulting fragment, containing the phosphorylatable scFv(D1.3) gene, was gel-purified and ligated into SfiI/Not1 digested pDN227.

pDN255

ScFv(HyHEL-10) with a $(Gly_4Ser)_3$ linker (Neri *et al.*, 1995, J.. Mol. Biol., 246, 367-373) was subcloned into the Sfi1/Not1 sites of pDN227.

pDN268

Using pDN227 as template and LMB3 and FLAGFORHIS (SEQUENCE ID NO 14) (5' TGA CTG GAA TTC TTA TTA GTG GTG ATG GTG ATG GTG CTT GTC ATC GTC GTC CTT GTA GTC 3') as primers, a His_6 tail was appended by PCR to the C-terminal extremity of the phosophorylation site and of the FLAG tag of pDN227. The SfiI/EcoR1 digested PCR product was then cloned into the SfiI/EcoR1 sites of pDN22 (Neri *et al.*, 1995, J. Mol.Biol., 246, 367-373).

pDN269

The calmodulin gene was PCR amplfiied from pDN152 using primers FLAGCAMBACK (SEQUENCE ID NO 15) (5' GAC TAC AAG GAC GAC GAT AAG GCT GAC CAA CTG ACA GAA GAC CAG 3') and LMB2. The gene of scFv(D1.3),

tagged with phosphorylation site and FLAG, was PCR amplified from pDN227 with primers LMB3 and FLAGFOR (SEQUENCE ID NO 16) (5' CTT GTC ATC GTC GTC CTT GTA GTC 3'). The two PCR products were then purified using Wizard PCR Preps (Promega), then PCR assembled (25 cycles, 94°C 1 min, 55°C 1 min, 72°C 2 min) using LMB2 and LMB3 as primers. The assembly band was gel-purified, SfiI/EcoR1 digested and subcloned into SfiI/EcoR1 digested pDN22 (Neri *et al.*, 1995b), yielding plasmid pDN269 which appends at the C-terminal extremity of the scFv(D1.3) gene the phosphorylation site, FLAG and the calmodulin gene.

E.coli TG1 cells (Gibson, 1984, Ph.D. Thesis, University of Cambridge Studies on the Epstein-Barr virus genome) were transformed with these constructs. 1 litre cultures (2xTY + 0.1% glucose + 100 μg/ml ampicillin).

Phosphorylation of recombinant proteins has been successfully achieved by tagging them with the recognition sequence (RRASV or RRASL) of cAMP-dependent protein kinase (Li *et al.*, 1989, PNAS USA, 86, 558-562). In this Example, the anti-lysozyme scFv(D1.3) antibody fragment (McCafferty *et al.*, 1990, Nature (London), 348, 552-554) is used as a model (vector pDN5 in Figure 1) to test whether this strategy would work with antibodies.

From pDN5, two E.coli soluble expression vectors (pDN30 and pDN31) were cloned by PCR, which append at the C-terminal extremity of scFv(D1.3) the phosphorylation sequence RRASV and the myc-tag (Munro and Pelham, 1986 Cell, 46, 291-300; Marks *et al.*, 1991 J. Mol. Biol., 222, 581-597), with or without a seven aminoacid spacer (Fig. 1 and described in Example 1 above). The two constructs allowed the secretion of several milligrams scFv per liter of culture which could be purified on a hen egg lysozyme affinity column as described in Example 2 (Ward *et al.* 1989, Nature (London) 341, 544-546). However, the purified antibodies had lost the tag, as demonstrated by their inability to be phosphorylated and by negative results in ELISA using the anti-myc antibody 9E10 (Marks *et al.*, 1991, J. Mol. Biol., 222, 581-597). Addition of protease inhibitors like soybean trypsin inhibitor and phenyl methyl sulfonyl fluoride (but not benzamidine) to the cell culture during antibody secretion allowed the detection of only a very weak ELISA signal in the supernatants. This susceptibility to proteolysis is attributed to the presence of positively charged Arg residues in the tag. Thus,

casein kinase II peptidic substrates were used as tags for protein phosphorylation.

The C-terminal extremity of scFv(D1.3) was tagged with the phosphorylatable sequences DDSDDDEE (SEQUENCE ID NO 1) (pDN223, Fig. 1 described above) and DDDSDDDflag (SEQUENCE ID NO 2) (pDN227; flag = DYKDDDDK (SEQUENCE ID NO 4); Hopp *et al.*, 1988). Tagging antibody fragments at the C-terminal extremity does not impair antigen binding (Ward *et al.*, 1989, Nature (London), 341, 544-546). pDN223 and pDN227 are general vectors for phosphorylatable antibody expression, allowing the subcloning of recombinant antibody gene at the Sfi1/Not1 sites (Fig. 1).

A phosphorylatable peptidic sequence as linker was cloned between VH and VL of scFv(D1.3) (pDN232) and the anti-lysozyme scFv(HyHEL-10) (Lavoie *et al.*, 1992, J. Immunol., 148, 503-513). The linker sequence DDDSSSDDDSDE (SEQUENCE ID NO 17) was chosen which, combined with the extremity of VH and VL genes, provides six putative phosphorylatable serines (Marin *et al.*, 1986, Eur. J. Biochem., 160, 239-244; Kuenzel *et al.*, 1987, J. Biol. Chem., 262, 9136-9140; Fig. 1).

Finally, casein kinase II sequences were incorporated both at the linker and at the C-terminal position (pDN250; Fig. 1). This construct contains seven putative phosphorylation sites. Before phosphorylation, the kinase substrate sequences contribute with 18 negative charges (at pH 7.0) to the final isoelectric point of the recombinant antibody.

EXAMPLE 2: Antibody expression and purification

Soluble expression of phosphorylatable antibodies was obtained with similar protocols for volumes ranging between 5 ml and 1 litre. Described hereinbelow is a procedure normalised for one litre. Ten ml of an overnight culture of the bacteria harbouring the appropriate plasmid were grown in 2xTY medium containing 1% glucose and 0.1 mg/l ampicillin, inoculated into 1 litre fresh 2xTY medium containing 0.1% glucose and 100 μg/ml ampicillin and grown at 37°C for approximately 2 hours, up to $A^{600} = 0.8$. The cells were then induced by addition of IPTG (final concentration = 1 mM), and grown at 20°C for 24 hours. The final suspension was then centrifuged. The resulting supernatant was filtered (0.45 μm cut-off) and applied onto a hen egg lysozyme-sepharose column (Ward *et al.*, 1989, Nature (London), 341, 544-546). The column was

then washed with at least 20 column volumes of TBS (50 mM Tris-HCl, pH 7.4, + 100 mM NaCl), then 20 column volumes of TBS + 0.5M NaCl. The antibody was eventually eluted with 100 mM triethylamine in 3 ml fractions, in tubes containing 1ml 1M Tris-HCl, pH 7.4. The antibody was concentrated (if needed) using 2.5 ml Centricon-30 tubes and analysed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970, Nature (London), 227, 680-685) on Homogeneous 20 SDS Phast gels (Pharmacia).

All the constructs could be expressed and purified on a lysozyme-sepharose affinity column, with yields of several milligrams per liter of culture supernatant. The protein preparation from pDN249, however, showed the presence of two principal bands, of which only one corresponded to the expected molecular weight. A BIAcore analysis (Jönsson *et al.*, 1991, BioTechniques 11, 620-627) showed that all the antibody samples had a kinetic dissociation constant (k_{off}) towards hen egg lysozyme similar to the one of the recombinant antibody without phosphorylatable tag (data not shown).

EXAMPLE 3: Phosphorylation

(a) Trace labelling for gel-electrophoresis analysis

Antibody phosphorylation was typically performed in a reaction mixture containing 19μl buffer A (50 mM Tris, pH 7.4, 200 mM NaCl, 12 mM $MgCl_2$), 1μl ATP (1 mM), 1μl high activity γ-(^{32}P)-ATP (NEN; 3000 Ci/mmol, 10mCi/ml), 5 μl of protein solution at (0.1-5.0 mg/ml) and 0.3 μl casein kinase 2 (10 u/μl, Promega). As a control, dephosphorylated casein (Sigma) was used. The reactions were incubated at room temperature for 20-30 minutes, then 4 μl of each reaction was mixed with 2 μl 3x SDS gel loading buffer (65 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 2.3% SDS, 10% glycerol) and run on a high-density SDS Phast gel (Pharmacia). After 20 minutes, the lower part of the gel (containing unreacted ATP) was cut. The gel was then fixed, stained with Coomassie, destained and dried at 70°C under vacuum. The dried gel was then imaged with a Phosphoimager (Molecular Dynamics), which was also used to quantitate the intensity of the bands.

The same protein samples analysed by SDS-PAGE and Coomassie (all compounds at roughly 0.3 mg/ml, except casein at 0.5 mg/ml), were phosphorylated using casein kinase II and γ-[^{32}P]-ATP, and analysed by gel electrophoresis. All antibody samples,

except DN223, were better substrates than casein. Samples DN227 and DN250 showed excellent incorporation of ^{32}P and good stability. DN232 and DN249 were very well phosphorylated, but were proteolysed at the level of the linker after labelling. Sample DN223 was also proteolysed, but its efficiency of phosphorylation was clearly worse than that of casein. The results of labelling were quantified using a Phosphorimager (Molecular Dynamics), and are summarised in Table 1, below.

TABLE 1

Efficiency of ^{32}P labelling of recombinant antibodies

Protein°	% incorporation*	% incorporation as scFv†
DN223	15	0
DN227	470	90.3
DN232	650	10.9
DN249	240	18.2
DN250	250	90.7
casein	100	-

° All protein samples were at approximately 0.3 mg/ml, except casein (0.5 mg/ml)

* The percent of ^{32}P incorporation is relative to the casein (0.5 mg/ml)

† This percent corresponds to the ratio of intensity of the scFv band, divided by the total intensity of the phosphorylated antibody bands.

EXAMPLE 4: Gel-retardation assays

Gel retardation (or "band-shift") is a useful technique for determining protein-DNA (Müller et al., 1988, Embo J. 7, 4299-4304; Carey, 1988, PNAS USA, 85, 975-979) and protein-protein affinities (Carr and Scott, 1992. Trends Biochem Sc., 17, 246-250). Gel retardation has been used for the study of antibody-antigen complexes (Neri *et al.*, 1995, J. Mol. Biol., 246, 367-373). A prerequisite for this technique is the very sensitive detection of one of the two molecular species involved in the binding equilibrium. In the case of antibody-antigen binding, the high-sensitivity detection of antibody bands in gels

allows one to titrate the antibody (at a fixed concentration, smaller than the K_d of the complex) with increasing amounts of antigen. Under these conditions, the K_d for the complex is approximately equal to the concentration of antigen which gives semisaturation of the antibody. This is strictly true only for those antibody-antigen complexes whose dissociation is negligible during electrophoresis time.

Fluorescent labelling of recombinant antibodies at a position that does not interfere with antigen binding has been described (Neri *et al.*, 1995, J. Mol. Biol., 246, 367-373). For practical applications, this technique is currently limited to K_d values larger than 100 pM by the sensitivity limit of fluorescence detection. Site-specific phosphorylation allows one to label recombinant antibodies to very high specific activity, without impairing antigen binding, and should allow one to extend the scope of gel-retardation techniques for the study of antibody-antigen interactions.

ScFv(D1.3) (sample DN250) and scFv(HyHEL-10) (sample DN255) were labelled with ^{32}P and a gel retardation experiment was performed. The results are illustrated in Figure 3. D1.3 is semi-saturated at lysozyme concentrations close to 10 nM, in agreement with that published before. HyHEL-10 is half-titrated between 0.1 and 1 nM lysozyme. These results confirm that ^{32}P-labelled recombinant antibodies fully retain their immunoreactivity, and that very high antibody-antigen affinities can be measured by gel retardation.

A gel retardation analysis of DN232 and DN249 was performed and confirmed that the samples, although proteolysed at the level of the linker to Fv fragments, stilll retain full immunoreactivity (data not shown).

^{32}P-labelled recombinant antibody samples were incubated with increasing amounts of lysozyme for 30 minutes at room temperature, then mixed with 6x native gel buffer (4 g sucrose + 25 mg bromophenol blue in 10 ml water) and run on a high-density native Phast gel (Pharmacia), fixed, stained with Coomassie, destained, dried at 70°C under vacuum and imaged.

EXAMPLE 6: Phosphorylation of scFv(HyHEL-10) (Sample DN 255)

a) Trace-labelling to establish the number of phosphorylatable sites per antibody construct. The construct DN255 (prepared as described in Example 1) (1nmol)

was treated with ATP (12.5nmol), [^{32}P]-γ-ATP (1μCi) and casein kinase 2 (1.25μl, 125U) in a total volume of 187.5μl of buffer A (see Example 3). After incubating at 20°C for 30 min or 24hr, 10μl samples were taken and added to 100μl bovine serum albumin (BSA, 2mg/ml) in phosphate-buffered saline (PBS) immediately followed by 100μl of 20% trichloracetic acid (TCA). The precipitated protein was collected on GF/C filters (Whatman) and the ^{32}P counted in 2ml of Optiphase scintillation fluid. Total counts were obtained by adding 10 μl of the reaction mixture to 2 μl of Optiphase. The number of phosphate groups incorporated per scFv molecule was found to be 0.85 at 30min and 0.95 at 24 hr.

b) High specific activity labelling. The antibody construct DN255 (1 mg in 680 μl buffer A) and casein kinase 2 (5 μl, 500U) was added to 700μCi of [^{32}P]-γ-ATP (9.5 μl > 7.000 Ci/mmole, Cat. No. 35020 from ICN Biomedicals, diluted with 85.5 μl of buffer A) and the reaction mixture incubated at 20°C for one hour. The unreacted ATP was then removed on a PD-10 column containing Sephadex G-25M which had been prewashed in PBS containing bovine serum albumin (BSA, 2mg/ml) and then equilibrated with PBS. In this experiment, 655 μCi was recovered, of which 64.8% was bound to the construct giving a product containing about 450 μCi/mg (Fig 2).

EXAMPLE 7: Stability of phosphorylated constructs in human plasma

A sample of ^{32}P-labelled DN255 (10μl, containing 2.3μCi, prepared as described in Example 6(b)) was added to fresh human plasma (1 ml) containing penicillin/streptomycin (10 μl, Sigma, P 0906). The mixture was incubated at 37°C and triplicate samples (20 μl) were taken at 1, 24, and 48 hours. Acid precipitates were obtained, collected on glass fibre filters and counted, and showed no significant loss of radioactivity during the course of the experiment (92-103%). Gel electrophoresis demonstrated that all the radioactivity continued to be associated with the correct MW of about 28.000 Daltons.

EXAMPLE 8: Preliminary Mouse Study

In a preliminary study with non-tumour bearing mice, it was observed that the ^{32}P labelled construct pDN321 was excreted mainly intact (molecular weight approximately

28.000) in the urine at 3 hours post injection, together with some ^{32}P-labelled peptide of molecular weight approximately 10.000 but only traces of free ^{32}P-phosphate.

In another experiment, plasma samples taken from nude mice three hours post-injection had a similar pattern following electrophoresis and autoradiography though with rather more proteolysed construct and no detectable free ^{32}P-phosphate. pDN321 is the single-chain anti-CEA antibody "MFE-23" cloned in the SG1/Not1 sites of pDN268.

These results are important because they indicate that ^{32}P-labelled CK 2-receptor constructs are not being significantly dephosphorylated by circulating phosphatases.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Andrew Malcom Creighton

(B) STREET: 68 Millway Mill Hill

(C) CITY: London

(E) COUNTRY: Engand

(F) POSTAL CODE (ZIP): NW7 3QY

(A) NAME: Keith Eric Britton

(B) STREET: 122 Hertford Road De Beauvoir Town

(C) CITY: London

(E) COUNTRY: England

(F) POSTAL CODE (ZIP): N1 4LP

(A) NAME: Medical Research Council

(B) STREET: 20 Park Crescent

(C) CITY: London

(E) COUNTRY: England

(F) POSTAL CODE (ZIP): W1N 4AL

(ii) TITLE OF INVENTION: Radiolabelled Proteins

(iii) NUMBER OF SEQUENCES: 17

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: GB 9501940.2
(B) FILING DATE: 01-FEB-1995

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9509984.2
(B) FILING DATE: 17-MAY-1995

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9518645.8
(B) FILING DATE: 08-SEP-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Asp Asp Ser Asp Asp Asp Glu Glu
1 5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Asp Asp Asp Ser Asp Asp Asp
1 5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Gly Ala Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Asp Tyr Lys Asp Asp Asp Asp Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GTCCTCGCAA CTGCGGCCGC AAGAAGGGCA AGTGTTGAAC AAAAACTCAT CTCAGAA 57

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GTAAAACGA CGGCCAGT 17

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GTCCTCGCAA CTGCGGCCGC AAGAAGGGCA AGTGTTGGCG GTTCTTCCGG CTCC 54

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GCGGCCGCAG GCGGTTCTTC CGGCTCCTGT GAACAAAAAC TCATCTCAGA AGAGGATCTG 60
AATTAATAAG AATTC 75

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CAGGAAACAG CTATGAC 17

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TGACTGGAAT TCTTATTATT CTTCATCGTC GTCGGAATCG TCATCTGCGG CCGCCCGTTT 60
GATCTCGAG 69

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TGACTGGAAT TCTTATTACT TGTCATCGTC GTCCTTGTAG TCATCGTCGT CGGAATCGTC 60
ATCTGC 66

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GTCACCGTCT CCTCAGACGA TGACTCTTCC TCTGATGACG ATTCTGACGA AGACATCGAG 60
CT 62

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CGATGTCTTC GTCAGAATCG TCATCAGAGG AAGAGTCATC GTCTGAGGAG ACG 53

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TGACTGGAAT TCTTATTAGT GGTGATGGTG ATGGTGCTTG TCATCGTCGT CCTTGTAGTC 60

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GACTACAAGG ACGACGATAA GGCTGACCAA CTGACAGAAG ACCAG 45

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CTTGTCATCG TCGTCCTTGT AGTC 24

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Asp Asp Asp Ser Ser Ser Asp Asp Asp Ser Asp Glu
1 5 10

CLAIMS

1. A structurally modified binding protein wherein the binding protein is fused to a peptide sequence capable of acting as a substrate for a casein kinase II enzyme.

2. A structurally modified binding protein according to Claim 1 wherein the peptide sequence is a synthetic peptide sequence.

3. A structurally modified binding protein according to Claim 1 or 2 wherein the peptide sequence is 5-20 residues in length and comprises at least one phosphorylatable residue and at least two negatively charged residues to the left (NH_2 terminal), right (COOH terminal) or left and right of the phosphorylatable residue less than 5 or 5 residues spacing away from the phosphorylatable residue.

4. A structurally modified binding protein according to Claim 3 wherein the phosphorylatable residue in the peptide sequence is threonine or serine.

5. A structurally modified binding protein according to Claim 4 wherein the phosphorylatable residue in the peptide sequence is serine.

6. A structurally modified binding protein according to Claim 3, 4 or 5, wherein the negatively charged residues in the peptide sequence are glutamate or aspartate residues.

7. A structurally modified binding protein according to Claim 6 wherein the negatively charged residues in the peptide sequence are aspartate residues.

8. A structurally modified binding protein according to any of Claims 3-7 wherein there is more than one phosphorylatable residue in the peptide sequence.

9. A structurally modified binding protein according to any of Claims 3-8 wherein the negatively charged residues in the peptide sequence are 3 and 1 residues spacing away from the phosphorylatable residue.

10. A structurally modified binding protein according to any of Claims 3-8 wherein the negatively charged residues in the peptide sequence are 5 residues spacing away from the phosphorylatable residue.

11. A structurally modified binding protein according to Claims 9 or 10 wherein the negatively charged residues in the peptide sequence are on the COOH terminal side of the phosphorylatable residue or residues.

12. A structurally modified binding protein according to Claim 9 or 10 wherein the negatively charged residues in the peptide sequence are on the NH_2 and COOH terminal sides of the phosphorylatable residue or residues.

13. A structurally modified binding protein according to Claim 12 wherein the peptide sequence contains a negatively charged residue to the left (NH_2 terminal) of the phosphorylatable residue or residues which is 3 residues spacing away and a negatively charged residue to the right (COOH terminal) of the phosphorylatable residue which is 5 residues spacing away.

14. A structurally modified protein according to any preceding claim wherein the peptide sequence is 5-15 residues in length.

15. A structurally modified binding protein according to Claim 14 wherein the peptide sequence is

DDSDDDEE : SEQUENCE ID NO 1

DDDSDDD : SEQUENCE ID NO 2.

16. A structurally modified binding protein according to any preceding claim wherein the binding protein is an antibody or antigen binding fragment thereof.

17. A structurally modified binding protein according to Claim 16 wherein the antibody is a monoclonal antibody or antigen binding fragment thereof.

18. A structurally modified binding protein according to Claim 17 wherein the monoclonal antibody or antigen binding fragment thereof is humanised.

19. A structurally modified binding protein according to any preceding claim wherein the peptide sequence is attached to the linked region of the binding protein.

20. A structurally modified binding protein according to any one of Claims 1-18 wherein the peptide sequence is attached to the C-terminal end of the binding protein.

21. A structurally modified binding protein according to any preceding claim for use in therapy.

22. A pharmaceutical composition comprising a structurally modified binding protein according to any preceding claim and a pharmaceutically acceptable diluent or carrier.

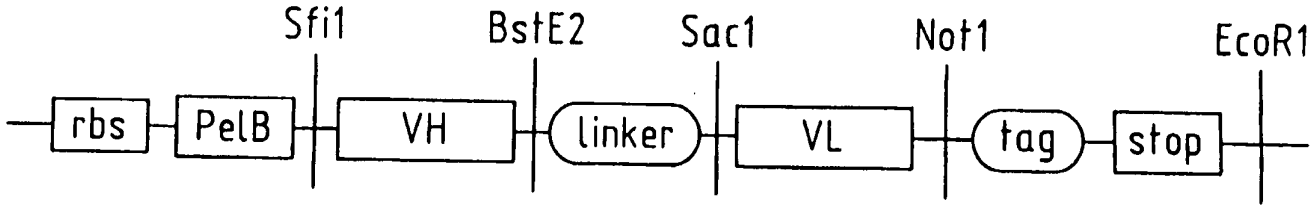
23. The use of a structurally modified binding protein according to Claims 1-19 for the manufacture of a medicament for the treatment of a patient in need of radioimmunotherapy.

24. A method of treating a patient in need of radioimmunotherapy comprising the administration of a therapeutically effective amount of a structurally modified binding protein according to any of Claims 1-20.

25. A structurally modified binding protein according to any of Claims 1-20 for use in analytical applications.

26. A structurally modified binding protein according to any of Claims 1-20 labelled with ^{32}P.

27. A structurally modified binding protein according to any of Claims 1-20 labelled with ^{33}P.



construct	Ab	V_H	linker	V_L	tag
pDN 5	D1.3	..SS --	GGGGSGGGGSGGGGS	-- DIE..	myc
pDN 30	D1.3	..SS --	GGGGSGGGGSGGGGS	-- DIE..	RRASVmyc
pDN 31	D1.3	..SS --	GGGGSGGGGSGGGGS	-- DIE..	RRASVGGSSGSCmyc
pDN 223	D1.3	..SS --	GGGGSGGGGSGGGGS	-- DIE..	DDDSDDDEE
pDN 227	D1.3	..SS --	GGGGSGGGGSGGGGS	-- DIE..	DDDSDDDflag
pDN 232	D1.3	..SS --	DDDSSSDDDSDE	-- DIE..	myc
pDN 249	HyHel-10	..SS --	DDDSSSDDDSDE	-- DIE..	myc
pDN 250	D1.3	..SS --	DDDSSSDDDSDE	-- DIE..	DDDSDDDflag
pDN 255	HyHel-10	..SS --	GGGGSGGGGSGGGGS	-- DIE..	DDDSDDDflag
pDN 268	D1.3	..SS --	GGGGSGGGGSGGGGS	-- DIE..	DDDSDDDflag-His_6
pDN 269	D1.3	..SS --	GGGGSGGGGSGGGGS	-- DIE..	DDDSDDDflag-cam

Fig.1

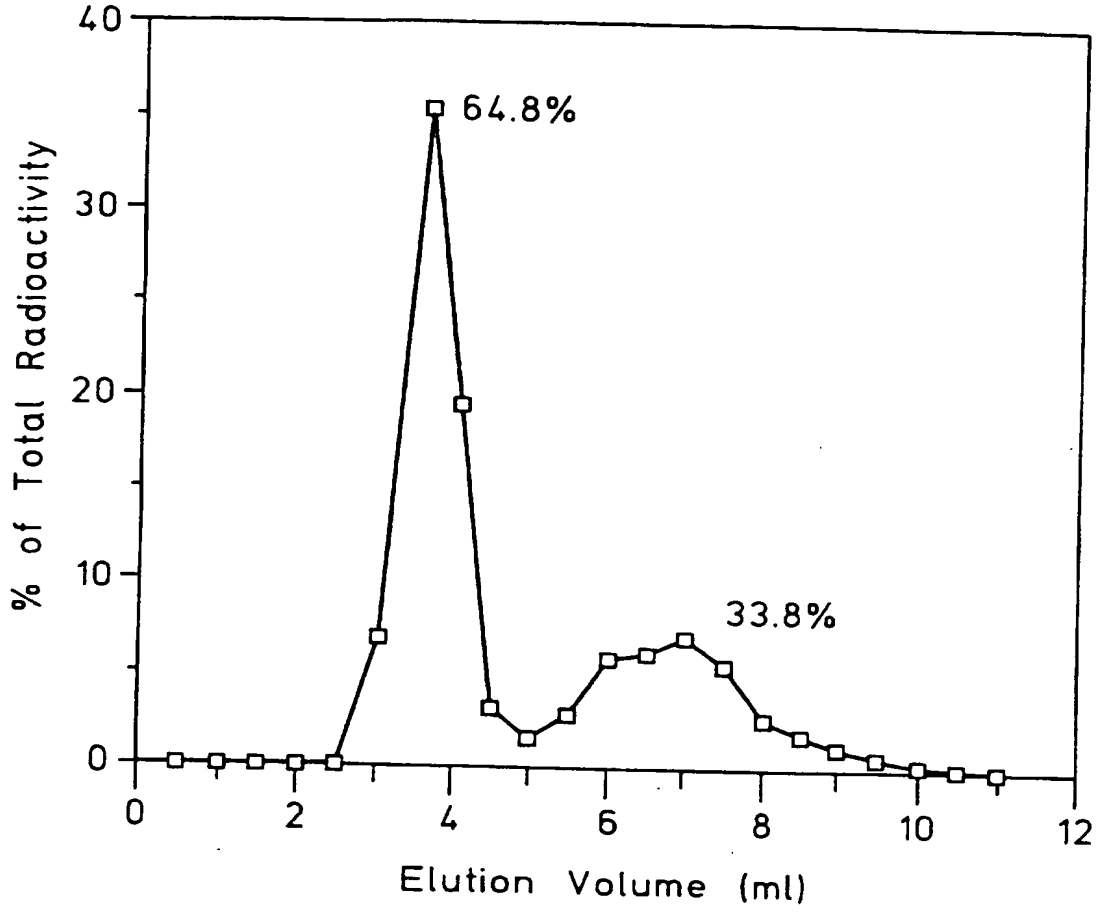

40
30
20
10
0
% of Total Radioactivity
64.8%
33.8%
0
2
4
6
8
10
12
Elution Volume (ml)

Fig.2

INTERNATIONAL SEARCH REPORT

Inte mal Application No
PCT/GB 96/00148

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K19/00 C07K16/30 C07K16/40 A61K51/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GB,A,2 262 528 (BRITISH TECH GROUP) 23 June 1993 cited in the application see the whole document	1-27
Y	GB,A,2 186 579 (FOXWELL BRIAN MAURICE JOHN;PARKER PETER; CREIGHTON ANDREW MALCOLM) 19 August 1987 cited in the application see the whole document	1-27
Y	WO,A,90 11289 (NAT RES DEV) 4 October 1990 cited in the application see the whole document	1-27

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
24 April 1996	10.05.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Sitch, W

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INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/GB 96/00148

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,USA, vol. 86, pages 558-562, XP002001476 LI ET AL: "CREATION OF PHOSPHORYLATION SITES IN PROTEINS:CONSTRUCTION OF A PHOSPHORYLATABLE HUMAN INTERFERON ALPHA" cited in the application see the whole document ---	
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INTERNATIONAL SEARCH REPORT

Information on patent family members

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